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Gait: Taira's group and others suggested linking four or five ribozymes together in a single transcript (Chen et al 1992, Ohkawa et al 1993). Have you tried similar experiments?

Rossi: No. We started with two, and we are concentrating our efforts on getting this through clinical trials.

Gait: In vitro do you get any improvements with two tandem ribozymes?

Rossi: Yes. What is probably happening is that the binding of RNA on one side opens up downstream sites, so there is more cleavage of that message than if you had the same concentration of each independent ribozyme alone. This may also be the case *in vivo*.

Monia: If the clinical trials are successful what do you anticipate the hurdles would be in the manufacturing process of these ribozymes?

Rossi: The major problem is manufacturing the vector because it has to be free of replication-competent retrovirus. This has been studied by the Chiron corporation. Ribozyme Pharmaceuticals Inc. became involved in this project and it became a way of testing a ribozyme in a clinical trial. They use a dog cell line to produce the retroviruses, which completely eliminates the potential for recombinant retroviruses. Murine cell lines are not used to produce these retroviruses because they have endogenous retroviruses to begin with, which complicates matters. In terms of scale-up, I'm not sure how valuable it would be from a financial point of view for a company because the treatment will be limited to those that can afford it. Ultimately, we would like to carry out this type of transduction using marrow from patients that have been ablated by putting transduced cells into the ablated marrow, which will increase the survival potential of the transduced cells. This is expensive and it will depend on defining the patient population in sufficient numbers that make it financially feasible.

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Exogenous application of ribozymes for inhibiting gene expression

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Abstract. Sequence-specific inhibition of gene expression is an attractive concept for the development of a new generation of therapeutics. Two alternatives can be envisaged for the introduction of ribozymes into cells: endogenous or exogenous delivery. In the latter, the ribozyme is prepared by chemical synthesis or transcription and delivered to the cell either unaided or with the help of liposomes. A problem with this approach is the abundance of RNases in the serum, and thus the stabilization of the ribozyme is necessary but without the impairment of catalytic efficiency. This has been achieved by several groups by 2'-modification of the pyrimidine nucleosides and the introduction of a few phosphorothioates at the termini. The selection of ribozyme-accessible sites on the target and the attachment of cholesterol and peptides to the ribozymes will be discussed. Examples of the application of these modified ribozymes in cell cultures will be presented, including the inhibition of expression of the multiple drug resistance gene, after unaided as well as liposome-aided delivery, and studies of animal models demonstrating the potential of this particular application strategy.

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The sequence-specific inhibition of gene expression has attracted much attention as a potential therapeutic agent. This concept has certain advantages over today's strategies as it is aimed at preventing the synthesis of a particular, harmful protein rather than using a drug that acts on it. As many disease-causing proteins, such as oncogenes, differ from the wild-type by only a few mutations, it is often difficult to develop a drug that acts specifically on the mutant protein without affecting the wild-type protein and, therefore, causing serious side-effects. The sequence-specific inhibition of translation offers the opportunity to prevent the expression of the mutant protein specifically.

The concept of this strategy was first conceived by using antisense oligodeoxynucleotides approximately 15 nucleotides in length that are complementary to a segment of the mRNA for the protein in question. The successful application of this strategy is discussed throughout this book and has also been reviewed elsewhere (Wagner 1994). A further development was the use of antisense RNAs gleaned from nature, a mechanism used by some organisms to

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regulate gene expression. Examples of this strategy are also dealt with throughout this book. The advent of catalytic RNA, i.e. ribozymes, offered the opportunity to tailor them for the sequence-specific inhibition of gene expression. There are several reviews describing progress in this area (Marschall et al 1994, Rossi 1995, Birikh et al 1997a, Eckstein & Lilley 1996). I will concentrate in this chapter on our work with the small, and therefore accessible by chemical synthesis, hammerhead ribozyme.

The hammerhead ribozyme

The hammerhead ribozyme (Fig. 1.) can in principle cleave any RNA that contains a $U_{16.1}H_{17}$ sequence at the cleavage site and where the annealing arms of the ribozyme are complementary to the target RNA to form helices I and III.

There are in principle two modes of delivery of ribozymes to cells: endogenous and exogenous delivery. The first consists of cloning the ribozyme gene into a vector for transfection or transduction so that the ribozyme is transcribed in the cell. The second attempts to get the preformed ribozyme into cells, often with the aid of a carrier, such as cationic liposomes. Thus, this mode of application is identical to that used with oligodeoxynucleotides, and therefore suffers from the same problems. My group follows this strategy.

Just as with oligodeoxynucleotides, the ribozymes have to be stabilized against nucleases when they are administered by exogenous delivery. We have solved this

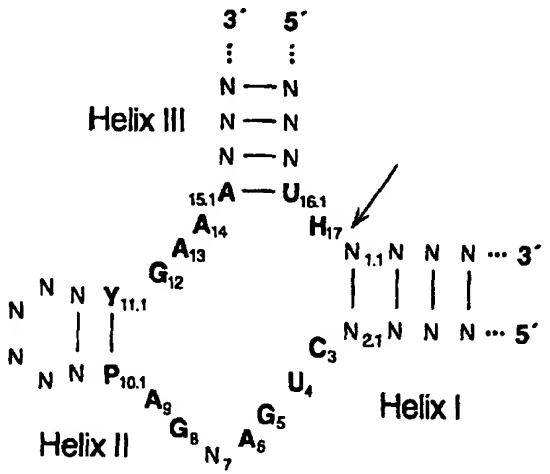

FIG. 1. Consensus sequence of the hammerhead ribozyme. Numbering system is according to Hertel et al (1992). Conserved nucleotides are in bold. N = any nucleotide; P = A or G; Y = U or C; H = A, C or U; B = C, U or G; V = G, A or C. The arrow represents the position of cleavage.

problem by substituting all pyrimidine ribonucleotides by 2′-fluoro or 2′-amino derivatives in combination with some terminal phosphorothioates (Pieken et al 1991, Heidenreich et al 1994). Others have chosen alternative 2′-derivatives (Paolella et al 1992, Beigelman et al 1995). All these constructs are stable for several days in serum and in nuclei suspensions (Heidenreich et al 1996).

Another problem familiar to those working with oligodeoxynucleotides is the selection of suitable sites on the target RNA. As mRNAs are known to form secondary structures, it is easy to imagine that segments which contain a triplet susceptible to cleavage might not be accessible to the ribozyme. Computer fold programs are available to calculate the structure with the lowest free energy; however, it is generally agreed that the predictive power of these calculations has limitations. An experimental approach to find such sites might therefore be preferable. Lieber & Strauss (1995) have developed such a method by using a ribozyme, with randomized annealing arms, directed against a GUC triplet in human growth hormone mRNA. This approach was successful for cytoplasmic extracts from cells overproducing this RNA, and one of the selected ribozymes was further developed to inhibit gene expression in cell culture and in mice (Lieber & Kay 1996). We have chosen another route by using a completely randomized oligodeoxynucleotide of 10 nucleotides (dN_{10}) in conjunction with RNase H to identify potential ribozyme cleavage sites on human acetylcholinesterase mRNA (Birikh et al 1997b). This assay identified several sites on a transcript accessible for the dN_{10} and the RNase H. Cleavable triplets were found in the vicinity of five of these sites, and ribozymes directed against these were active in cleaving the transcript. The most active ribozyme was 250-fold more active than the best designed on the basis of the MFold computer program. Experiments to verify that these sites are also accessible *in vivo* are in progress.

Hammerhead ribozymes cleave short substrates with annealing arms not longer than five to seven nucleotides with k_{cat}/K_m efficiencies of $10–100 \times 10^6$ min^{-1} M^{-1}. Unfortunately, however, long substrates, such as transcripts, are cleaved with efficiencies that are several 100-fold lower, which is almost entirely due to the k_{react} values as the K_m values are similar to those observed with short substrates (Heidenreich et al 1994, Hendrix et al 1996) This effect is presumably due to a slow annealing step, and also to slow product release. Fortunately, certain RNA-binding proteins have been shown to improve the efficiency by 30-fold by catalysing both of these steps (Herschlag 1995). Nuclear proteins have shown similar effects (Heidenreich et al 1995). However, it is not clear what these rates are inside a cell and what rates are necessary for an efficient inhibition of gene expression. Obviously, a ribozyme could also inhibit gene expression by an antisense effect due to the complementarity of the sequences to form the ribozyme–substrate complex (Fig. 1). The importance of the catalytic effect can easily be checked by using a ribozyme where catalysis is inactivated by replacing one of the essential nucleotides in the core region.

Delivery of ribozymes to cells is still problematic. As this obstacle is identical to that for the delivery of oligodeoxynucleotides, it is not surprising that the ribozyme field

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follows developments in the oligodeoxynucleotide field closely. Cationic liposomes seem to be the carriers of choice at present (Leonetti et al 1993). Uptake of oligodeoxynucleotides has been shown to be facilitated by the attachment of cholesterol (Letsinger et al 1989, Krieg et al 1993). We have taken this lead to synthesize ribozymes, directed against the HIV long terminal repeat (LTR) and luciferase, which contain cholesterol that is coupled via disulfide, amide or carbamide bonds (S. Alefelder, B. Patel, F. Eckstein, unpublished observations 1996). The kinetic characteristics of these ribozymes and their power to inhibit gene expression in cell culture are at present under study. Another problem with the use of ribozymes is their slow cleavage rate of long substrates. It is believed that this is due to the slow annealing step. Recently, a peptide consisting to a large extent of lysine and arginine residues was found to accelerate the hybridization of single-stranded oligodeoxynucleotides (to form double-stranded oligodeoxynucleotides) (Corey 1995). We have attached this peptide to one of our ribozymes making use of the 2'-amino group at one of the nucleosides near the 3' terminus. We have shown previously that such an amino functionality reacts readily and specifically with aryl isothiocyanates and even better with alkyl isocyanates (Sigurdsson & Eckstein 1995, 1996). Cleavage kinetics of a long transcript with the ribozyme conjugate showed that the cleavage rate was only marginally improved, i.e. a fivefold improvement (B. Patel, F. Eckstein, unpublished observations 1996). However, these results suggest that other peptides might be coupled to a ribozyme to direct compartment localization.

Several examples show that the exogenous application of chemically modified ribozymes is a viable concept. Expression of the MDR-1 gene, which is responsible for multiple drug resistance, has been inhibited by such a ribozyme in a human cell line restoring antibiotic sensitivity (Kiehntopf et al 1994). The effect of long-chain unmodified and short-chain chemically modified HIV1 *tat*- and LTR-directed ribozymes was compared in another study (Hormes et al 1997). The explanation of this differential effect is not entirely clear at present. Proviral HIV1 DNA and the ribozymes were co-microinjected into either the nucleus or the cytoplasm of human cells. Interestingly, the long-chain ribozymes, when injected into the nucleus, inhibited viral replication, but this inhibition did not occur when they were injected into the cytoplasm. The inverse effect was seen with the short ribozymes. Even more encouraging are results obtained with *in vivo* animal models. Jarvis et al (1996) delivered a chemically modified ribozyme directed against c-*myb* with cationic liposomes to rat aortic smooth muscle cells, and this led to the inhibition of serum-induced cell proliferation. A chemically modified ribozyme directed against amelogenin, when injected into the developing molar teeth of newborn mice, temporarily prevented biomineralization (Lyngstadaas et al 1995). In another animal model Flory et al (1996) describe the decrease of stromelysin mRNA upon injection of a chemically modified ribozyme into rabbit knee joints. Stromelysin, a metalloprotease, is considered to be a mediator in arthritic disease. Surprisingly and encouragingly, in both these *in vivo* experiments the ribozymes reached their targets without the help of a carrier.

In summary, numerous examples illustrate that the concept of using preformed, chemically modified ribozymes for exogenous delivery for the inhibition of gene expression represents a viable and promising strategy, although the method requires considerably more work to be of general use.

Acknowledgements

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DISCUSSION

Rossi: In your random oligonucleotide RNase H experiments, where your colleague designed the ribozyme based upon those sites most accessible to RNase H, what were the arm lengths of the ribozymes? Were they comparable to the oligonucleotide or were they longer?

Eckstein: They were longer. I showed you the results for a random 10 mer, but we also did the experiment with a 15 mer and there was no difference. The ribozymes designed on the basis of these experiments had seven nucleotides in each arm, thus they were a bit longer than the random 10 mer oligonucleotide.

Rossi: The RNase H treatment is performed at 37 °C, and although the fold program is performed at a certain temperature, it doesn't take into account the binding of the oligonucleotide to various regions of the substrate. Therefore, it's difficult to predict if an AU-rich ribozyme, for example, is going to bind to a looped region at 37 °C. We have had some experience with this approach. We mixed randomized arms of ribozymes with N-labelled substrate, although this didn't work too well because of the shear numbers of ribozyme variants we had to deal with, and when we did a fold program to direct ribozymes to a particular site we found that the predicted open sites

were only accessible to ribozyme pairing at 4 °C, in the case of short-arm ribozyme, because of the stability limitations of the short-armed hybrids.

Eckstein: The fold program that the Ribozyme Pharmaceuticals Inc. (RPI) group uses is more sophisticated because it takes into account, for each stretch of the RNA, its involvement in the global secondary structure and the stability of possible secondary structures not corresponding to the optimal overall structure. It also considers the ribozyme structure (Christoffersen et al 1994).

Gait: I was somewhat confused over your conjugation procedure. You mentioned attaching cholesterol or peptides through 2′-amino functional groups but you also said that you used 2′-amine groups on several positions simultaneously, so you must have changed your ribozymes somewhat.

Eckstein: The 2′-amino uridine is in the centre. However, we attach cholesterol or the peptide to ribozymes where the 2′-amino group is present only once close to the 3′ terminus.

Gait: Is it reasonably efficient to make conjugates using that technique?

Eckstein: Yes, although it's not 100% efficient. We have to purify the conjugate using HPLC or FPLC.

Gait: Why is there a fivefold increased catalytic efficiency with peptide-conjugated ribozymes? Were there changes in K_m?

Eckstein: In these single turnover experiments the increased efficiency is mainly due to an increased K_{cat}. It is debatable whether the on-rate is the rate-limiting step because the on-rate actually represents multiple steps. I would say that the on-rate is not rate limiting *per se*, rather it is the conformational change between the on-rate and the subsequent steps. The factor of five in these experiments is close to the S.D. so I wouldn't like to interpret this in any sort of mechanistic way.

Cohen: Even though you retain activity with these multiple chemically modified groups, are you changing either the conformation or the interactions?

Eckstein: I assume not, although I don't have any data on this. We don't interfere with the binding to the target, and the bottom line is that the catalytic efficiency remains the same.

Letsinger: The objectives of the chemotherapy and antisense approaches are different. Is this because the antisense gene therapy approach could never result in a cure for HIV, for example?

Rossi: For HIV, which is a chronic infectious disease, the gene therapy approach could result in a cure. If the viral infection is minimized by having cells protected over a long period of time it is possible that the virus can be cleared, although this is clearly going to be difficult to prove. However, this is the goal of the gene therapy approach. The exogenous delivery approach would have to be maintained continually throughout. It also has the same potential, i.e. if the immune system can reconstitute itself in the disease it's possible to clear the virus. We should not focus entirely on therapy because there are other situations where ribozymes, and indeed antisense oligonucleotides, are useful: for example, the exogenous application of ribozymes can be used to study gene function.

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Gait: To take that a step further, there should now be the possibility of comparing antisense and ribozyme approaches to the same target. Has anyone looked at this?

Iversen: In our rat liver model we looked at the efficacy of the 2'-O-allyl hammerhead ribozyme. We found that although the clearance of ribozymes was reasonably rapid, the effective molecules resided in the liver longer than traditional antisense compounds.

Eckstein: The RPI group also reported that ribozymes were more efficient than antisense oligonucleotides in their system (Jarvis et al 1996). However, if you are looking at a ribozyme site and then you direct your oligonucleotide against the same site this will bias your perception. A fairer comparison would be if one also optimized for the antisense oligonucleotide site.

Gewirtz: Is there any reason to think that one should necessarily be better than the other, precisely for the reason you just mentioned?

Eckstein: No. It is not necessarily clear that one should be better than the other.

Iversen: One consideration might be that, in terms of the specificity of the ribozyme, at least using the conservative mutation approach, this compound is absolutely inert in the rat liver. Another consideration is exposure measured by the area under the curve. It doesn't matter if the compound falls apart, as long as it isn't rapidly cleared into the urine. The race is against clearance versus breakdown.

Matteucci: In terms of whether we should use antisense oligonucleotides or ribozymes, many of us believe that oligonucleotides bind tightly to proteins in cells, such that there is a sequestration phenomenon and a small amount is free for hybridizing. Is there any evidence that ribozymes are different? Has anyone microinjected a fluorescein-tagged ribozyme to determine its cellular location and whether it is covered with protein immediately upon entering the cell?

Rossi: One experiment that may be possible to do with a ribozyme is to incorporate a signal into the non-base pairing sequences that will allow it to go back out into the cytoplasm. For instance, the U1 small nuclear (sn) RNA has a sequence motif that is involved in transporting it from the nucleus into the cytoplasm. Also, the adenovirus UA1 RNA is a small cytoplasmic RNA that has a structural motif which binds a protein that allows it to exit the nucleus.

Krieg: A related question, from someone who doesn't work on ribozymes, is what is the limiting factor in ribozyme efficacy? Is it finding the right sequence to target? Is it proteins that for some reason bind to a particular ribozyme structures?

Eckstein: But we can't even answer these questions for antisense oligonucleotides. Certainly, one of the limiting factors is that at the beginning we are not really certain which target sites are accessible. For ribozymes it has been shown that nuclear proteins improve RNA cleavage (Heidenreich et al 1995).

Gerwirtz: What are the ionic requirements for magnesium ions, in terms of comparing ribozymes *in vivo* and *in vitro*?

Eckstein: Ribozymes do have a requirement for magnesium ions. The optimal concentration *in vitro* is 10 mM, whereas the free concentration of magnesium ions in cells is much lower, of the order of 200–300 μM.

Gewirtz: An oligonucleotide might work better *in vivo* because it does not have those kinds of requirements.

Eckstein: We know little about the distribution of free magnesium ions, but the ribozymes work, and we assume that this has something to do with cleavage of the RNA.

Gewirtz: But there are others who assume that this is because the flanking arms provide an antisense effect.

Eckstein: One of our control experiments involved inactivating the ribozyme by changing one of the essential nucleotides in the core. If it was working via an antisense mechanism then we would not have observed this effect.

Gewirtz: But you said that you couldn't target your antisense molecule necessarily to the same region that you're targeting the ribozymes, so those may not be the best controls for those experiments.

Rossi: It was only the catalytic core of the ribozyme that was changed and not the mechanism by which it binds.

Caruthers: One of my concerns is the low rate of catalysis for ribozymes. Is this a serious problem?

Eckstein: It doesn't seem to be, but then we know little about the catalytic efficiency within the cell. There are proteins that increase efficiency by a factor of about 30, and there may be other as yet undiscovered proteins that improve on this.

Rossi: RPI have made some modifications of the catalytic core region that result in increased catalytic efficiency, and probably also increase the rate of the cleavage step by up to 50-fold (Burgin et al 1996). They are obtaining K_{cat} values that are approaching those of a protein. In terms of site-specific cleavage activity, the ribozyme is equivalent to *Eco*RI.

Eckstein: There have been many reports where people have increased the catalytic efficiencies of ribozymes. However, when you look at the data carefully the K_m value has increased by the same amount as the increase in K_{cat}. In my opinion there is little scope for large improvements.

Agrawal: Is anything known about the cellular uptake of ribozyme RNA or linear RNA compared to DNA, and have there been any stability comparisons?

Vlassov: We investigated the efficiency of oligonucleotide uptake and found that it was more efficient for long oligonucleotides. The data suggest participation of a protein receptor in the oligonucleotide uptake.

Lebleu: There are some old studies on poly I–poly C, which is a synthetic double-stranded RNA, and these reported that uptake is relatively inefficient. Microinjected poly I–poly C is 10^4-fold more active (in terms of interferon induction) than poly I–poly C in the cell culture medium. This indicates how much can be gained by appropriate delivery.

Stein: Scavenger receptor will pick up RNA, as opposed to DNA, and this receptor although common, is not ubiquitous. Therefore, the rate at which RNA enters the cell is going to be both highly sequence dependent and cell type dependent, i.e. dependent on the density of scavenger receptor, and it is therefore not possible to generalize.

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Eckstein: In terms of exogenous delivery, the RNA is highly modified and this structure may not be recognized by scavenger receptor.

Pieken: It's not clear to me how we can talk about RNA uptake without talking about stability. The rate of free RNA degradation is so fast.

Monia: I have a question about RNA oligonucleotides, as opposed to ribozymes. Under rare circumstances, we have observed a reduction in RNA levels with a RNA-based oligonucleotide, provided we stabilize it. We think that we're utilizing enzymes such as RNase III. In your controls have you seen similar reductions in RNA levels with RNA oligonucleotides?

Eckstein: We haven't, but almost certainly because we haven't looked for it.

Cohen: I have a question about the rate of the reaction. The ribozyme reaction is a transphosphorylation, but most enzyme reactions are proton transfers, which are much faster. Therefore, doesn't this suggest that there is an intrinsic rate-limiting step when comparing ribozymes to other enzymes?

Eckstein: We normally see a rate of one per minute for the chemical step. The problem is that for many enzymes people don't look at the chemical step. For instance, the rate-limiting step for restriction enzymes is not the cleavage reaction, rather it is often product release.

Inouye: You mentioned that free RNA inside the cell is not stable. However, this may not always be true. For example, we've been working on a particular mRNA in *E. coli*, in which the RNA is much less stable than other cellular mRNAs at 37 °C, and this mRNA is only induced at low temperature. This induction is controlled by mRNA stability at 37 °C, even if the gene is transcribed constitutively. The half-life of the mRNA is only 12 seconds or less. This is why the gene cannot be expressed at 37 °C. However, when we incorporate three point mutations near the Shine-Dalgarno sequence the half-life of the mRNA increases to 30 min, even at 37 °C. The mRNA has a 159 bp 5′ untranslated region. It is not yet known how the stability of the mRNA in the cell is being controlled.

Eckstein: Are these point mutations at either end?

Inouye: No. They are in the middle of the gene, near the ribosome-binding site.

Toulmé: I have a question about ribozyme specificities. Ribozyme target sequences are significantly longer than those that have been used in antisense oligonucleotide studies, and the longer the antisense sequence the more likely non-specific effects will be observed because of binding to partially complementary sequences. Is there any evidence that the structure of ribozymes is so strong that it cannot unfold, or is it reasonable to think that this structure can open and bind to a region that will generate non-specific effects?

Eckstein: I am not aware of any studies on this.

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